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(54) Title: HUMANIZED C-erbB-2 SPECIFIC ANTIBODIES (57) Abstract Immunoglobulins specific for the polypeptides encoded by the <i>erb</i> oncogene are provided. The amino acid sequences of the immunoglobulins are modified so as to contain human immunoglobulin amino acid sequences in substantially all regions of the immunoglobulin other than the complementarity determining regions. Also provided are nucleic acid sequences encoding a humanized immunoglobulin, replication vectors containing such nucleic acid sequences, and host cells containing the vectors, for the expression of humanized immunoglobulins. Therapeutic and diagnostic compositions for administration to patients are also taught.		

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Humanized C-erbB-2 specific antibodies

Field of the Invention

The present invention related to the field of molecular biology in general, and the field of chimeric immunoglobulins in particular.

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Background

The present invention relates to altered immunoglobulin molecules in which at least part of the complementarity determining regions in the light or heavy chain variable domains have been replaced by analogous complementarity determining region(s) from a murine antibody specific for c-erbB-2.

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The c-erb-2 (HER-2) oncogene may be expressed in human breast carcinoma cells but is either not expressed, or expressed at much lower levels, in non-cancerous cells. It is thus of interest to provide antibodies specific for the erb encoded protein. Antibodies specific for this protein may be used to assay for the presence of cells expressing the erb encoded protein, or may be applied to patients
15 so as to specifically bind to carcinoma cells either for diagnostic, imaging or therapeutic purposes.

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It is of interest to produce monoclonal antibodies specific for antigens that are expressed in much higher amounts in tumors, such as erb. Monoclonal antibodies have numerous advantages over polyclonal antibody preparations to the
20 same antigen. These advantages include higher specificity and the ability to reproducibly generate large quantities of the antibody of interest. Since most monoclonal antibodies are of murine or non-human origin, their administration into human patients is a significant problem. Introduction of non-human antibodies into human patients may have a variety of adverse effects. Such
25 adverse effects include the development of an antibody response directed to many portions of the administered monoclonal antibody, such as HAMA (human anti-mouse antibody). Additionally, the antibody may fail to interact with other portions of the human immune system, e.g., a murine antibody Fc region may not interact with human Fc receptors, thus resulting in the absence of the desired

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immune response to cells displaying the antigen of interest, or may fail to activate complement. Attempts to produce human monoclonal antibodies specific for antigens of interest have proven to be difficult for several reasons, including the lack of good fusion partners for human cells, ethical problems associated with immunizing human patients to obtain lymphocytes, as well as difficulty in obtaining human lymphocyte donors. Because of the difficulties associated with obtaining human antibodies specific for the desired antigen, it is of interest to provide for "humanized" murine antibody specific for the antigen, i.e., an antibody that contains primarily human amino acid sequences and some of the variable region sequence of a conventional murine antibody specific for the desired antigen.

Humanized antibodies (or more generally, humanized immunoglobulins) have at least three potential advantages over murine antibodies for use in human therapy.

Because the constant region portion is human, a humanized antibody may interact better with other parts of the human immune system (e.g., destroy the target cells more efficiently by complement dependant cytotoxicity or antibody-dependent cellular cytotoxicity).

The human immune system should not recognize the humanized portions of the humanized immunoglobulin as foreign, and therefore the immune response against an injected humanized immunoglobulin should be less than the immune response against an injected totally murine immunoglobulin.

Injected murine antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of human antibodies (Shaw, et al., J. Immunol., 138:4534-4538 (1987)). It is possible that injected humanized immunoglobulins will have a half life more like that of human immunoglobulins, thus allowing smaller and less frequent doses of therapeutic immunoglobulins to be administered to the body with the same or better outcome.

Numerous attempts have been made to circumvent the problems associated with administering non-human monoclonal antibodies to humans by modifying non-human antibodies so as to replace non-human sequences with amino acid

sequence derived from human antibodies. Several publications, patents and patent applications disclose humanized antibodies and methods for their production by recombinant DNA technology. See for example, European Patent Application EPA-0-239-400, Recombinant Antibodies and Methods for Their Production; PCT Application WO89/09622, IL-2 Receptor-Specific Chimeric Antibodies; European Patent Application EPA 0-338-745, Method for Producing Recombinant DNA Proteins; and European Patent Application EPA 0-332-424, Chimeric Antibodies Directed Against Human Carcinoembryonic Antigen. Typically, murine monoclonal antibodies are raised against an antigen of interest, the immunoglobulin genes encoding the antibody of interest are then extracted from the hybridoma genome, sequenced, and genetically manipulated so as to replace non-human constant region sequences with human constant region sequences. Such "chimeric" antibodies contain murine variable regions and human constant regions.

Although others, e.g., Winter in EPA-0-239-400, have replaced human hypervariable sequences with murine hypervariable sequence specific for an antigen of interest in order to obtain humanized "hyperchimeric" antibodies specific for an antigen, such teachings provide no expectation of success for attempts to produce humanized antibodies (or derivatives thereof) specific for c-erbB-2 or any other given antigen. Antigen combining sites have complex 3-dimensional structures that are in part dependant on the primary amino acid sequence of the variable region of immunoglobulins. The general procedure and concerns associated with producing humanized, chimeric and hyperchimeric antibodies can be found in Antibody Engineering, edited by Borreback, W. H. Freeman and Co. Publishers. Thus changing several amino acids within the variable region of an immunoglobulin would not be expected to have an predictable effect on the structure (and consequently on antigen binding properties) of the variable region.

Summary of Invention

The subject invention provides for humanized immunoglobulin molecules (and derivatives thereof) specific for c-erbB-2. Nucleic acid sequences encoding these immunoglobulins, and cells for the expression of the humanized immunoglobulins are also provided for.

The subject invention also provides for methods of using the humanized immunoglobulins to diagnose and treat cancer. Cells for the production of humanized c-erb-2 immunoglobulins and in vitro synthesis methods for these immunoglobulins are also taught.

Description of Figures

Figure 1 provides the nucleotide sequence (total 1554 base pairs) encoding a humanized anti-erbB2 specific Fab fragment (SEQ ID:No. 1) derived from the hypervariable regions (also referred to as complementarity determining regions, of abbreviated CDR) of murine anti-c-erbB-2 monoclonal antibody 520C9. The humanized light and heavy chains of the Fab fragment have been modified to contain an E. coli phoA leader sequence. A plasmid comprising the nucleotide sequence of Figure 1 is pLW187. The E. coli strain containing pLW187 is referred to as TLW170-1 in this application.

The sequence of Figure 1 (SEQ ID:No. 1) may be divided into the following subsequences:

1-143bp phoA promoter and leader;

144-779bp Humanized Heavy Chain;

144-233 FR1 (human parent TSYC 1147-28);

234-248 CDR1 (mouse 520C9);

249-290 FR2 (human parent TSYC 1147-28);

291-342 CDR2 (mouse 520C9);

343-437 FR3 (human parent TSYC 1147-28);

438-455 CDR3 (mouse 520C9);

456-488 FR4 (human parent TSYC 1147-28);

489-779 Human Heavy Chain Constant Region

780-821KT3 tag;
822-893bp phoA leader;
894-1538bp Humanized Light Chain;
894-966 FR1 (human parent TSYC 1150-38);
967-998 CDR1 (mouse 520C9);
999-1043 FR2 (human parent TSYC 1150-38);
1044-1064 CDR2 (mouse 520C9);
1065-1160 FR3 (human parent TSYC 1150-38);
1161-1187 CDR3 (mouse 520C9);
1188-1220 FR4 (human parent TSYC 1150-38);
1221-1538 Human Light Chain Constant Region;
1539-1554 XhoI/BamHI Cloning Sites;

Figure 2 represents a general scheme for humanizing murine monoclonal antibodies by overlapping PCR.

Figure 3 represents the scheme and PCR primers used to produce a humanized heavy chain immunoglobulin.

Figure 4 represents the scheme and PCR primers used to produce a humanized light chain immunoglobulin.

Figure 5 represents the scheme used to introduce a phoA leader sequence and promoter sequence in front of a humanized immunoglobulin.

Figure 6 represents the scheme used to correct an unintentional sequence error made during the production of a humanized light chain derived immunoglobulin made according the scheme described in Figure 4.

Eight primers and four templates are used in three round of overlapping PCR. LW01 is the primer specifically designed for amplifying the 5' end of the phoA sequence. LW16 and LW17 is a set of complementary junction primers which sit at the end of the phoA leader and the beginning of the coding sequence in FR1. The template pSYC1087 contains the phoA promoter and, when used with primers LW01 and LW17, will yield a fragment (1) containing a 5' HindIII site and a 3' end bearing sequence for the beginning of PR1. CLC27 is a primer

which anneals to the end of FR2 and part of CDR2. PCR using LW16 and CLC27, with the template "08-11" will result in a product (II) containing correct FR1, CDR1, FR2 and CDR2. Complementarity of LW16 and LW17 allow annealing of fragments I and II in a second round of PCR, producing fragment (V).

CLC26 is a primer which contains CDR1 and partial FR2 sequence but does not reach amino acid #43. CLC29 is complementary to CLC30 and anneals to FR4 as well as the beginning of the human light chain constant region. PCR with CLC26 and CLC29, using template "38-18" yields a product (III) containing correct CDR1, incorrect FR-2 containing alanine at amino acid #43, and correct CDR2, FR3, CDR3, and FR4. Since "38-18" has an incorrect constant region, "38-17" is used as template for primer CLC30 and LW20. This fourth PCR product ((V) contains correct CDR3, FR4, and human light chain constant region. Complementarity of CLC29 and CLC30 at CDR3 allow fragments III and IV to anneal for the second round of overlap PCR, producing fragment VI.

PCR fragments V and VI are complementary at framework 2 except at amino acid residue #43, where in fragment V the residue is threonine (ACC) and where in fragment VI, the residue is alanine (GCC). Annealing of these two fragments during the final round of PCR will yield products which due to the differing nature of its templates, will bear amino acid alanine or threonine at residue 43.

Description of Specific Embodiments

A. Definitions

An immunoglobulin molecule may be divided into several regions. An immunoglobulin molecule may comprise one or more polypeptide chains, i.e. a multi-polypeptide immunoglobulin. IgG, for example, consists of 2 heavy (H) chains and 2 light (L) chains. An immunoglobulin chain typically comprises variable regions and constant regions; the H chain of IgG contains one variable region and one constant region, each constant region has a different sequence, and the (L) chain has one variable and one constant region. The constant region of an H chain can be further subdivided into 3 domains, each of which forms a separate

compact tightly folded 3 dimensional unit. The L and H variable regions are similarly folded into separate compact units. The variable region of an immunoglobulin chain (H and L) may be further divided into three hypervariable regions (also called complementarity determining regions, abbreviated CDR) and four framework regions (abbreviated FR). The framework regions separate the hypervariable regions from each other in the linear amino acid sequence. Compared to the hypervariable or CDR regions, the framework regions comprise amino acids that do not vary as much between immunoglobulins produced by the same organism and are reasonably fixed in 3-dimensional space. Framework region amino acids sequences may exhibit more variation within an organism than constant region amino acid sequences. Hypervariable regions, on the other hand, vary to a much greater extent between individual immunoglobulin molecules produced by the same organism, and are less well fixed in 3-dimensional space. The hypervariable regions are believed to form a major part of the antigen binding site of an antibody. The framework regions of the immunoglobulins are believed to form two opposing beta-pleated sheets, which are the basic structural element of the domain. These strands of the beta sheet are connected by loops of polypeptide chain which are thought to contain the hypervariable regions of the variable region of an immunoglobulin. Interactions between certain framework and CDR residues may influence the folding of the protein, particularly the hypervariable loops, and affect the ability of the antigen binding site to recognize antigen.

Constant regions of an immunoglobulin are located distal or C-terminal to the variable region. Constant regions may be of a light chain class including kappa and lambda chain constant regions (and the various subclasses thereof), or may be of a heavy chain class including the heavy chain constant regions of IgG, IgM, IgA, IgD, and IgE antibodies (and the various subclasses thereof).

"Operably joined" refers to a juxtaposition such that normal function of the components can be performed. Thus, a coding sequence "operably joined" to expression control sequences refers to a configuration wherein the coding sequences can be expressed under the control of these sequences. Such control may be direct, that is, a single gene associated with a single promoter, or indirect,

as in the case where a polycistronic transcript is expressed from a single promoter.

"Control sequence" and "expression control sequence" refer to a DNA sequence or sequences necessary for the expression or regulation (transcriptional or translational) of an operably joined coding sequence in a particular host organism. The control sequences, that are suitable for procaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, a transcription terminator, and possible other as yet poorly understood sequences. Eucaryotic cells are known to utilize control sequences, which include promoters, polyadenylation signals, enhancers, silencers, and the like.

The term "functional pair" when used with reference to variable regions (humanized or otherwise) intends a set of 2 variable regions (one derived from the L chain and one from the H chain) that form an antigen combining site in an antibody. Functional pairs of variable regions specific for an antigen of interest may be found in the variable region from the heavy chain and the variable region from the light chain of an antibody specific for the antigen of interest. Examples of functional pairs of variable regions include the polypeptides encoded by nucleotides 144-488 and nucleotides 894-1220 of Figure 1 (SEQ ID:NO1).

The term "functional proximity" when used with reference to variable regions (humanized or otherwise) intends that the functional pair of L and H variable regions be spatially located with respect to each other so as to form an antigen combining site.

The terms "heavy chain-derived" and "light chain-derived" when applied to immunoglobulins of the subject invention indicate that the variable region hypervariable sequences in the specified polypeptide are present on either the heavy chain or the light chain, respectively, of the antibody from which the hypervariable sequences of interest were originally discovered.

The term "humanized" as applied to immunoglobulins intends that at least a portion of the framework regions of an immunoglobulin are derived from human immunoglobulin sequences.

When sequences are said to be "human", all human alleles for the given sequence, in addition to the sequence specifically exemplified, are included.

Moreover, the subject invention contemplates that minor amino acid sequence changes, including substitutions, deletions, and insertions, typically in the range of about 1 to 5 amino acids, may be made to humanized immunoglobulins specific for c-erbB-2 without significantly altering the binding specificity of the immunoglobulin.

When a functional pair of variable regions are "specific" for a given antigen (or hapten), the antigen combining site formed by the functional pair is capable of binding to the antigen (or hapten) of interest more strongly than to randomly selected molecules.

10 B. General Description

The subject invention provides for immunoglobulin molecules with variable regions comprising the hypervariable regions of the anti-c-erbB-2 specific antibodies (in particular the murine monoclonal antibody 520C9), human framework regions, and human constant region sequences. In addition to providing for humanized c-erbB-2-specific immunoglobulin molecules, the subject invention also includes nucleic acid sequences encoding the humanized immunoglobulins, as well as cell host systems for the expression of humanized immunoglobulins.

Humanized immunoglobulins specific for antigens of interest may be obtained by preparing non-human, preferably murine, monoclonal antibodies against an antigen of interest, determining the amino acid sequence of the non-human antibody, preferably by isolating (by any of a variety of well known gene isolation techniques, including PCR) and sequencing cDNA sequences encoding the chains of the non-human monoclonal antibody of interest. The amino acid sequence of the hypervariable regions of the non-human monoclonal antibody may then be compared with canonical framework region sequences and hypervariable region amino acid sequences of human immunoglobulin variable regions so as to determine which amino acids must be changed to humanize the non-human sequence. Guidance for selecting the framework sequences and hypervariable sequences within the variable region of an immunoglobulin can be found in several

publications, including the article by Chothia, et al. Nature 342:878-879 (1989) and the book Antibody Engineering: A Practical Guide, Borrebaeck ed., W. H. Freeman and Co. publisher. By determining which sequences in the non-human immunoglobulin gene specific for the antigen of interest constitute framework regions and which constitute hypervariable regions, it becomes possible to synthesize a variety of humanized immunoglobulins having an antigen specificity similar to that of the non-human immunoglobulin that serves as the information source for the sequence regions.

Humanized variable regions specific for c-erbB-2 may be joined to a human constant region(s), or portions thereof. Joining of a humanized variable region to a constant region gives rise to a polypeptide in which the amino-terminal portion comprises the humanized variable region and the carboxyl-terminal portion comprises human constant region sequence. Such constructs may be referred to as "hyperchimeric". Humanized light chain-derived variable regions may be operably joined to light chain constant regions so as to form functional immunoglobulin chains. Similarly, humanized heavy chain-derived variable regions may be operably joined to heavy chain constant regions so as to form functional immunoglobulin molecules. In a preferred embodiment of the invention humanized light chain-derived variable region is operably joined to human light chain constant region, and humanized heavy chain-derived variable region is operably joined to a human heavy chain constant region(s).

In addition to providing for humanized immunoglobulins specific for c-erbB-2, the subject invention also provides for various polypeptides (and corresponding nucleic acid sequences) comprising portions of c-erbB-2 specific humanized immunoglobulins, in particular the variable region portion of the humanized immunoglobulin. Furthermore, the subject invention provides for various derivatives of c-erbB-2 specific immunoglobulin chains that comprise additional polypeptide sequences. These additional polypeptide sequences may have any of a variety of functions including, enzymes, toxins, antigenic tagging sequences, and the like.

Many modifications and variations of humanized variable region sequences

within the present demonstrative nucleic acid sequence of Figure 1 (SEQ ID: NO 1) are possible. For example, the degeneracy of the genetic code allows for the substitution of nucleotides (which can be used or optimized in accordance with codon usage patterns for expression in various host cells) throughout the polypeptide coding regions, as well as for the substitution of the translational stop signal specifically exemplified. Such sequences can be deduced from the known amino acid or DNA sequence of the 520C9 antibody chains (and the constant regions or antibodies of different classes) and can be constructed by conventional synthetic procedures. Such synthetic methods can be carried out in substantial accordance with the procedures of Itakura, *et al.*, 1977 Science 198:1056, Crea, *et al.* Proc. Nat. Acad. Sci. USA 75:5765 (1978). In addition, synthetic genes (and fragments thereof) and linkers can be synthesized either by using a Systec 1450a DNA synthesizer (Systec, Inc., 3816 Chandler Drive, Minneapolis, Minnesota) or an Applied Biosystems 380a DNA synthesizer (Applied Biosystems, Inc. 850 Lincoln Center Drive, Foster City, California 94404). Many other DNA synthesizing instruments are known in the art and can be used to make synthetic DNA fragments. Therefore, the present invention is no way limited to the DNA sequences specifically exemplified.

A preferred method of synthesizing nucleic acid sequences encoding humanized immunoglobulins is by means of overlapping PCR. The technique of overlapping PCR is described in the article by Horton, *et al.*, Gene 77:61-68 (1989) and in U.S. Patent No. 5,023,171. The technique of overlapping PCR may be used to produce a humanized immunoglobulin by performing overlapping PCR on cDNA sequence from a human immunoglobulin gene with the appropriate oligonucleotide primers.

In brief, the technique of PCR splicing by overlap extension is as follows. PCR oligonucleotide primer pairs are prepared. Each oligonucleotide primer comprises essentially two regions, a 5' region containing either sequences encoding a restriction endonuclease recognition site or a portion of complementarity determining region (CDR) from a non-human immunoglobulin gene of interest, and a 3' region complementary to a framework region adjacent to

the complementarity determining region to which the 5' portion of the primer is complementary. Sets of such PCR primers are prepared for each framework region of the human immunoglobulin gene that will furnish the human sequences for humanizing the non-human immunoglobulin gene of interest. The primers are constructed so that 5' and 3' regions that code within the same CDR region are overlapping. An advantage of using overlapping PCR to synthesize a nucleic acid sequence encoding a humanized non-human immunoglobulin sequence is in the minimization of restriction digest/ligation reactions and oligonucleotide synthesis reactions. The 5' CDR complementary regions of the PCR primers are complementary to the CDR complementary regions of PCR primers that have 3' regions complementary to adjacent framework regions. As indicated in Figure 2, repeated rounds of PCR give rise to a nucleic acid sequence comprising the parent human immunoglobulin sequence in all regions except for the complementarity determining regions, these regions being derived from the complementary determining regions of non-human immunoglobulin genes synthesizing an immunoglobulin specific for the antigen of interest. However, small portions of murine framework sequence (particularly the canonical residues described by Chothia, et al.) may be required in order to generate an active antigen combining site.

The humanized immunoglobulins chains of the subject invention are preferably utilized in the form of multi-polypeptide immunoglobulins comprising at least one humanized immunoglobulin variable region functional pair. In multi-polypeptide humanized immunoglobulins comprising variable region functional pairs, the variable regions of the functional pair are preferably localized in space (the pair members are in functional proximity to each other) so as to form an antigen combining site in which the antigen binding site is, at least in part, formed by the hypervariable regions of both the light chain-derived and heavy chain-derived hypervariable regions of 520C9 or other c-erbB-2 specific antibodies. However, the H chain variable region often contributes more than the L chain variable region in the interaction with antigen.

Single humanized immunoglobulin chains may be joined to each other so as

to form multi-polypeptide immunoglobulins by a variety of means. Such joining means include both ionic interactions and covalent bonds. The means of joining individual immunoglobulin chains so as to form a multi-polypeptide immunoglobulin are preferably, although not necessarily, by means of covalent linkage. The preferred means of covalent linkage is by means of disulfide bridges between cysteine residues located within the humanized immunoglobulin chains of interest; however, covalent linkage may also be effected through the use of cross-linking reagents such as dimethyl-3,3'dithiobispropionimidate, N-(4-azidophenyl) phthalamide, and the like. Humanized immunoglobulin chains may be joined so as to produce multi-polypeptide immunoglobulins molecules structurally analogous to antibodies or fragments thereof, including Fab fragments, Fab' fragments, F(ab')₂ fragments, Fabc fragments, Fd fragments, Fr fragments, Fv fragments, single chain Fv fragments, and the like. The available literature provides for methods for producing antibodies and fragments thereof from polypeptides synthesized by recombinant DNA and in vitro synthesis techniques.

The humanized immunoglobulins of the subject invention may be conjugated to a variety of therapeutic moieties. By therapeutic moieties it is intended a variety of compounds or atoms that find use in the treatment or detection of disease conditions. Compounds that find use in treating disease conditions include toxins (or active portions thereof) such as diphtheria toxin, ricin, or Pseudomonas exotoxin, enzymes, conventional drugs and prodrugs. In general, the humanized immunoglobulins of the subject invention may be derivatized by known method for conjugating therapeutic moieties to antibodies so as to produce highly specific drugs or imaging agents. Other therapeutic moieties of interest for conjugation to the humanized immunoglobulins of the subject invention include radio-opaque imaging agents. Therapeutic moieties also include radionuclides for use in imaging or in irradiating tissue, such as ⁹⁰Y, ¹⁸⁶Re, ¹⁸⁷Re, ⁶⁷Cu, ²¹¹At, ²¹²Pb, ²¹²Bi, ¹²⁵I, ¹³¹I, ¹²³I, and the like.

Humanized immunoglobulin sequences may be modified by the addition of a variety of a secretion signal sequences preferable joined to the amino terminus of the humanized immunoglobulin polypeptide. Secretion signal sequences (also

referred to as "signal sequences") serve to provide a signal to the secretion "machinery" of a cell to export polypeptides bearing such a sequence. The use of signal sequences to direct the cellular localization and/or export of polypeptides not naturally joined to the signal sequence, i.e., heterologous with respect to the signal sequence, is well known in the field of recombinant gene expression. Thus signal sequences may be used to simplify the process of purification of the subjects polypeptides from recombinant cell expression systems for several reasons, including obviating the need to lyse host cells and the need to produce subcellular fractions enriched for the polypeptide of interest. Leader sequences typically comprise a charged amino acid at the N-terminus followed by a short hydrophobic amino acid sequences. Leader sequences may be selected on the basis of the cellular expression system used to synthesize the humanized immunoglobulin polypeptide. Signal sequences are preferably selected so as to be removed either completely or substantially from the humanized immunoglobulin sequence of interest. The actual leader sequences employed will vary in accordance with the choice of cellular expression system selected. Although leader sequences are known to direct the localization of proteins in heterologous expression systems, i.e., host cells not naturally producing the protein that is the source of the signal sequence, it is preferable to use leader sequences from polypeptide naturally expressed in the cellular expression host. When expressing humanized immunoglobulins in bacterial systems, a PhoA, i.e., alkaline phosphatase, signal sequence is preferably used for expression in E. coli. Other bacterial protein signal sequences of interest include those from the ompA and pepB genes. For expression in mammalian cells, the use of an immunoglobulin leader sequence is preferred. For expression in yeast cells, an alpha factor leader sequence, among others, may be used as a signal sequence.

The humanized immunoglobulins of the subject invention may or may not contain "tag" amino acid sequences. Such "tag" sequences are short amino acid sequence, normally no more than 20 amino acids in length, preferable less than 15 amino acids in length. Tag sequences may be included in the amino acid sequence of the subject humanized immunoglobulins for the purpose of purifying, detecting

(or quantifying) the polypeptides of the subject invention by use of antibodies, including monoclonal antibodies, (or similar reagents) capable of specifically binding to the tag sequence. Tag sequences without attached immunoglobulin amino acid sequences may be synthesized in vitro using various well-known techniques, including commercially available polypeptide synthesis machines. The in vitro synthesized tag sequences may then be injected into suitable animals so as to induce an immune response directed to the tag sequence. Antibodies specific for a tag sequence of interest may also be prepared by immunizing an animal with a protein having a primary amino acid sequence that includes the tag sequence of interest. Tag sequences are preferably attached at or near the COOH terminal end of immunoglobulin molecules. A tag sequence of particular interest is a sequence recognized by the KT3 monoclonal antibody. An amino acid sequence recognized by KT3 is TPPPEPET. Another example of a tag sequence that may be inserted at the carboxy terminal (or internally as well) of an immunoglobulin sequence is the sequence EEEEYMPME. (Grussenmeyer, et al., Proc. Natl. Acad. Sci. USA 82, 7952-54 (1982)).

The humanized immunoglobulins of the subject invention may be expressed in cellular hosts after the sequences encoding the humanized immunoglobulins have been operably joined to expression control sequences. Nucleotide sequences for expression may be conveniently operably joined to expression control sequences by insertion into restriction sites in expression vectors. Expression vectors may contain expression control sequences located near useful restriction sites, and are typically replaceable in the host organism either as extra chromosomal elements, such as plasmids, or as an integral part of the host chromosomal DNA. Expression vectors may contain selectable markers, such as antibiotic resistance, to permit detection of those cells transformed with the nucleotide sequences of interest, see for example U.S. Patent No. 4,794,362, which is herein incorporated by reference.

The polypeptides of the subject invention may be expressed in a variety of cell types. The literature available to those skilled in the art describe numerous cellular expression systems for polypeptides of interest and expression vectors for

use in those systems. See for example Methods of Enzymology Vol. 185, Goeddel, Academic Press (1990). Humanized immunoglobulins may be recovered and purified from recombinant host cells using conventional techniques for recovery and purification of recombinantly produced proteins.

5 Nucleotide sequences encoding humanized immunoglobulins may be expressed in a variety of cells. Cells for expression may be either eukaryotic or prokaryotic. Prokaryotic hosts of interest include Bacillus subtilis, as well as other Bacilli, enterobacteriaceae, such as E. coli, as well as various Streptomyces, Salmonella, Serratia, and Pseudomonas species. Among prokaryotic host cells
10 species, E. coli is particularly preferred because of the great deal available literature dealing with expression in E. coli.

Other microbial organisms, such as yeast, may be used for expression of the subject humanized immunoglobulins. Saccharomyces cerevisiae is a preferred non-bacterial microbial expression host.

15 Other non-mammalian eukaryotic expression host cells of interest include insect cells that may be used with baculovirus expression systems.

In addition to the use of microbial, and invertebrate cells, mammalian cells grown in tissue culture may also be used to produce the polypeptides of the present invention. The polypeptides of the present invention may be expressed in
20 any mammalian cell system that may be used to express immunoglobulin polypeptides. Eukaryotic cells are preferred cellular hosts for the expression of subject polypeptides as opposed to non-mammalian cells, because of the numerous advantages associated with using mammalian cells, such advantages include suitable signal-sequence processing, glycosylation, secretion machinery and the
25 production of functional full-length immunoglobulins. Mammalian cells for use as expression hosts include CHO cell lines, various COS cell lines, HeLa cells, SP2/0 and the like.

Host cells for the expression of the polypeptides of the present invention may be genetically manipulated so as to produce one, or more humanized
30 immunoglobulins. When two humanized immunoglobulin chains are produced by the same cell line, it is of interest to produce a first immunoglobulin chain having

a humanized variable region that comprising one member of the functional pair of variable regions specific for c-erbB-2 and a second immunoglobulin chain comprising the other member of the same functional pair of variable regions for c-erbB-2. Thus, by producing cells genetically manipulated so as to express two
5 such humanized immunoglobulins chains, multi-polypeptide immunoglobulins comprising a functional c-erbB-2 antigen binding site may be produced either in vivo, or in the supernatants of cell cultures.

In addition to the production of humanized immunoglobulins by recombinant methods, automated equipment for the direct synthesis of polypeptides
10 disclosed herein is commercially available. Such equipment provides access to peptides of the invention, either by direct synthesis or by synthesis of a series of fragments that can be coupled using other known techniques.

The humanized immunoglobulins of the subject invention and pharmaceutical compositions thereof are particularly useful for parenteral
15 administration, e.g., subcutaneously intramuscularly or intravenously. The compositions for parenteral administration will typically comprise a solution of the humanized immunoglobulin dissolved in a physiologically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, physiological saline, 0.3% glycine, and the like. Solutions
20 for parenteral administration are preferably sterile and generally free of particulate matter. Compositions for parenteral administration may be lyophilized for convenient storage and rehydrated prior to use. These compositions for parenteral administration may be sterilized by conventional sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as
25 required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of humanized immunoglobulin in these formulations can vary widely, i.e., from less than about 0.5%, but usually at or at least about
30 1% to as much as 15% or 20% by weight and maybe selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of

administration selected.

The invention having been described, the following examples are offered to illustrate the subject invention by way of illustration, not by way of limitation.

Example 1

Overview of Production of Nucleic Acid Sequences Encoding Humanized Immunoglobulins

In overlap extension, PCR is performed with primers that have complementary 5' ends so that products from a first round of reactions can be mixed, melted, and reannealed to provide a template for the synthesis of longer extension products in a second round reaction. To humanize the murine antibody of interest, primers were designed whose 3' ends anneal to human frameworks, and whose 5' ends either contain cloning sites, or encode murine 520C9 CDR's. A general scheme for humanizing an antibody is shown in Figure 2. The first round of PCR utilizes DNA template from the selected human parent TSYC 1147-28 or TYSC 1150-38 and results in four individual fragments, each containing either human FR1, human FR2, human FR3 or human FR4, flanked by cloning sites and/or CDR's. The FR4 fragment is large because it also contains the first C_{H1} domain of the human constant region. The next round of PCR involves annealing CDR1 arms on first round products "A" and "B", and results in a longer fragment. "E", which consists of, in the 5' to 3' direction: a cloning site, human FR1, mouse CDR1, humanR2, and a primer generated CDR2 arm. The other round two reaction anneals mouse CDR3 arms on first round products "C" and "D", and produces a fragment containing human FR3, FR4 and constant regions. This second round product, "F", is flanked at its 5' end by mouse CDR2, and on the 3' end by another cloning site. The final round of PCR anneals the complementary CDR2 ends in fragments "E" and "F" and creates the complete humanized "G" fragment.

The "G" fragment is placed into an expression vector. Heavy and light chain "G" fragments undergo separate restriction digests for ligation into their own pUC vectors and are transformed into *E. coli* host DG101. Transformants are

screened for the presence of insert, and DNA from several potential candidates is sequenced. Clones with the correct sequence undergo another round of PCR with primers designed for incorporation of a phoA promotor/leader sequence in front of the antibody coding sequence (See Figure 5). The PCR product containing phoA-
5 antibody sequence is digested and cloned into a pBR322 based vector and transformed into *E. coli* host MM294. Transformants are screened, and potential candidates are identified and sequenced. Clones bearing the correct sequence of each chain are induced for expression on the putative humanized immunoglobulin and checked for expression by Western analysis. The final construct to produce a
10 humanized Fab fragment is made by combining the light chain coding sequence with the heavy chain coding sequence. This last process involves a PCR step and a restriction/ligation step.

Example 2

Selection of Human Parent Framework

15 Human frameworks, or "parents", were chosen by a "TFASTA" computer alignment which generated the ten best fits with human immunoglobulin sequences in the searched database. The selection was based on overall similarity of amino acid residues between the human and the original mouse 520C9 sequence. The human sequence database was produced by cloning approximately 100 heavy and
20 light chains of immunoglobulin genes from EBV transformed human cells, and subsequently sequencing the cloned genes. In addition, both L and H chain variable regions were sequenced from cDNA copied from human peripheral blood cells using primers that were designed for PCR amplification of the V regions into the adjacent constant domain these sequences and compared with the mouse
25 variable regions that were to be hyperchimerized.

The chosen human framework must not interfere with the presentation of the mouse CDRs to the target antigen. Chothia, *et al.* Nature 342:877-883 (1989) hypothesized that a small repertoire of "canonical" conformations of hypervariable regions exist and the structure of a given CDR is strongly influenced by a few
30 amino acid residues at key positions. Such canonical residues are found in CDR and FR regions. The human frameworks selected for each chain have canonical

residues similar to that of the mouse antibody.

According to the theory of Chothia, *et al.*, the heavy chain canonical residues of special importance are amino acids #26, 27, 29, 34, 55, and 94. In the mouse 520C9 heavy chain, these residues are, in order, Gly, Tyr, Phe, Met, Gly, and Arg. In the best fitting human heavy chain parent, TSYC1147-28, all canonical residues match except #34, which is an isoleucine instead of a methionine. No other human parent within the top five selected for overall sequence similarity matched better than TSYC1147-28. Thus, TSYC1147-28 was chosen as the human heavy chain framework parent for grafting with mouse heavy chain CDR's.

Light chain canonical residues of special importance are amino acids #2, 25, 29, 33, 48, 64, 71, 90, and #95. In the mouse 520C9 light chain, the residues are, in order, Ile, Ala, Ile, Leu, Ile, Gly, Tyr, Gln, and Pro. In the best fitting light chain human parent, TSYC1150-38, eight of nine residues match. Only residue number 71, where phenylalanine replaces tyrosine, does not match. The second best fitting light chain human parent, TSYC1150-08, matches at only six of the nine canonical residues. Amino acid #29 is leucine instead of isoleucine; #71 is phenylalanine instead to tyrosine; and #95 is phenylalanine instead of proline. Two human light chain frameworks, TSYC1150-38, and -08, were chosen for grafting of mouse light chain CDR's. Humanization of light chain has been completed with only TSYC1150-38 as parent.

Example 3

Design of Primers for Framework Grafting by Overlapping PCR

The expected sequences for heavy and light chain, from humanization of mouse 520C9 CDR's with human parent frameworks, is given in figure 1. Eight primers for each chain were designed based on the scheme presented in figure 4. Each primer contains two regions, one bearing sequence which anneals to human framework, and the other either containing a cloning site or bearing sequence which encodes for a mouse CDR. Half of the primers are upstream (located at the 5' end of the reaction) primers and the other half, downstream (located at the 3'

end of the reaction) primers. Each PCR reaction requires one upstream (front) and one downstream (back) primer. A short explanation of each primer follows (The abbreviations HC and LC refer to heavy chain and light chain, respectively):

CLC17(HC) or CLC24(LC):(upstream)

5' end contains cloning sites XhoI(HC) or SacI(LC);
3' end anneals to FR1.

CLC18(HC) or CLC25(LC):(downstream)

3' end anneals to FR1;
5' end encodes for CDR1.

CLC19(HC) or CLC26(LC):upstream

5' end encodes for CDR1.
3' end anneals to FR2.

CLC20(HC) or CLC27(LC):(downstream)

3' end anneals to FR2;
5' end encodes for CDR2.

CLC21(HC) or CLC28(LC):(upstream)

5' end encodes for CDR2;
3' end anneals to FR3.

CLC22(HC) or CLC29(LC):(downstream)

3' end anneals to FR3;
5' end encodes for CDR3.

CLC23(HC) or CLC30(LC):(upstream)

5' end encodes for CDR3;
3' end anneals to FR4, for extension through human constant

region.

SYC12(HC) or SYC324(LC):(downstream)

3' end anneals to the end of the HC or LC
constant region.

5' end contains cloning sites SpeI(HC) or XbaI(LC)

The heavy and light chain "G" fragments were digested with restriction enzymes in separate reactions and cloned into their own pUC vectors. Ligations were then transformed into DG101 host. Potential clones were identified by colony PCR or by miniprep analysis and were sequenced. The correctly humanized heavy chain in pUC was found in clone TCC19-4

Example 4

Design of Primers for phoA Incorporation 5' to the Humanized Heavy Chain

Four primers, W to Z, as shown in figure 5, are needed for the overlap extension PCR that incorporates phoA promoter and leader sequences in front of immunoglobulin coding sequences. Each primer in the figure is discussed below:

Primer W anneals to a portion of the plasmid pSYC1087 ahead of a region encoding for the phoA promoter and leader sequence. The primer can be divided into two parts. One part anneals to pBR sequence, the plasmid backbone, and the other anneals to restriction sites which precede phoA sequences in pSYC 1087.

W: (HC or LC)

LW01: 5'-GGGG ATCGAT AAGCTT GGG CTGCAG GTCGAC
pBR322 sequence HindIII PstI SalI

Primers X and Y together create an in-frame junction between the end of the phoA leader and the sequence encoding human amino acid #1 in the antibody. These primer sets are specific for each antibody. They are often modified to encode for amino acids that are missing from the human parent sequences. For example, immediately following, i.e. 3' to, the phoA sequences, the primers for the humanized 520C9 heavy chain junction region encode for the first six human consensus amino acids found in Kabat, et al., Sequences of proteins of immunological interest 4th ed, U.S. Dep. HHS (1987). It is necessary to supply these amino acids because the human library was created by primers which start at amino acid #7 of the heavy chain.

Y: (HC)

LW13: 5' ————— | GAG ATC CAA CTG GTG GAG | TCT GGG CCT GAG
GTG

LW14: GGA CAC TGT TTT CGG | CTC TAC GTT GAC CAC CTC | ———5'

X: (HC)

end of proA leader

1st six consensus human
HC a.a. from Kabat

start of FR1 in
TSYC 1147-28

Y: (LC)

LWI6: 5' —CCT GTG ACA AAA GCC | GAC ATC CAG ATG ACC CAG

LW17: GGA CAC TGT TTT CGG CTG TAG GTC TAC TGG GTC—5'

X: (LC)

end of phoA leader

start of FRI in TSYC 1150-38

521

Primer Z is a back primer whose 3' end anneals to the final nucleotides of

the human heavy or light chain constant region. Its 5' end contains cloning sites: either SpeI for heavy chain, or XbaI for light chain.

Z: (HC)

5 LW15: G TTG TTC CAC CTG TTC TTT | TGA TCA | CCC C—5'
 end of human heavy chain SpeI
 constant region

Z: (LC)

10 LW20: AAG TTG TCC CCT CTC ACA | ATT A | GAG CTC CCT AGG CGG—5'
 end of human light chain STOP XhoI BamHI
 constant region

15 The products were restriction digested with HindIII and SpeI and were cloned into a pBR based vector with an ampicillin selection marker. Downstream from the SpeI site, the vector carried the KT3 peptide sequence, NH₃-TPPPEPET-COOH, in frame with the heavy chain peptide and the B. thuringiensis crystal protein transcriptional terminator. Transformants in MM294 were screened for inserts, and several clones bearing insert were sequenced. LW156 contained correct phoA promoter and leader sequences and correctly encodes for the humanized "520C9-28" heavy chain.

Example 5

20 phoA Incorporation Ahead of Humanized Light Chain

The wrong template was inadvertently used to create the "G" fragment ("G" as in the Figure 2 scheme). The mistake was not realized until sequencing revealed that one of the humanized light chain "G" fragments for parent TSYC1150-38 matched that of the desired fused clone. Instead of starting over and
 25 creating a new "G" fragment using the correct template, the sequenced materials were modified. Amino acid alignment of sequenced clones with the fused "520C9-38" sequence indicated that clone "38-18" most resembles the desired sequence. It contained one error at amino acid 43 (in framework region 2, i.e., FR2), changing alanine to threonine and other errors in the constant region.

30 Overlap PCR was performed (see Figure 6) to create the correctly fused light chain sequence by using templates which were correct for different regions of the variable and the constant region, and at the same time, to incorporate the phoA

junction. pSYC1087 was used as the phoA template. "08-11" was used as a template for FR1, CDR1, and FR2 because it possessed the correct amino acid, threonine, at position #43. "38-18" was used as the template for FR2, CDR2, FR3, CDR3, and FR4. Another clone, "38-17" was used as template for the FR4 and the constant region. Existing primers were used to create products which would anneal to each other in second and third round extensions. Primer W, LW01, and light chain junction primer Y, LW17, were used to amplify pSYC1087. Light chain junction primer X, LW16, and CLC27 amplified "08-11". CLC27 was described earlier and anneals to FR2 and CDR2 regions. CLC26 and CLC20 amplified "38-18". CLC26 anneals to CDR1 and FR2, while CLC29 anneals to FR4 and part of the constant region. CLC30, a perfect complement to CLC29, and Z primer LW20 amplified "38-17" to provide the correct constant region.

In the second round, PCR products from pSYC1087 and "08-11" were melted, reannealed at the phoA-FR1 junction, and extended to create a longer fragment that contains correct sequence up to CDR2. Products from "38-18" and "38-17" were melted, reannealed at the CLC29-20 junction, and extended to form a longer fragment, which contains sequence from CDR1 to the end of the constant region. The latter second round fragment carries the incorrect amino acid #43, alanine, from its parent "38-18", and is the source of the problem for the final extension step. The two second round products melt and anneal at a large region encompassing CDR1, FR2, and CDR2, and extend to potentially form the final phoA-humanized light chain product. A problem occurs because there are two templates for amino acid residue 43, where one encodes for threonine and the other, for alanine. Because at least half of the templates for that position are incorrect, the same proportion of the final products will also bear that error.

The PCR products were digested with HindIII and XhoI and were ligated into a pBR322 based vector, similar to the one for the heavy chain, but minus the KT3 tag sequence. Transformants in MM294 were screened by colony PCR and by miniprep restriction analysis, and potential candidates were sequenced. Clone LW206 contains the correct phoA promoter and leader sequence, and correct

variable and constant region amino acid sequence for humanized "520C9-38" light chain.

Example 6

Construction of Humanized Fab

5 Plasmid from humanized heavy chain in the phoA expression vector, pLW144, was cut with NsiI and XhoI and purified. Separately, a PCR reaction was performed, using clone LW206 as template, to add a NsiI site ahead of the phoA leader sequence. Restriction digest of the PCR product with NsiI and XhoI resulted in a fragment of approximately 800bp ready for cloning behind the heavy
10 chain V gene. Ligation and transformation (TLW170) into MM294 E. coli host resulted in a large number of insert-bearing clones. Clones TLW170-1 and TLW170-3 were sequenced and confirmed to contain correct phoA, heavy chain, the KT3 tag, and light chain sequences.

Example 7

Purification of Humanized 520C9 E. Coli Expressed Fab

15 One liter of cell culture E. coli strain TLW170-1 was inoculated and induced for expression of the plasmid encoded proteins for about 8 hours.

Low Phosphate Induction:

A tube or flask containing Hi P medium [High Phosphate Medium (Hi P):
20 = Low phosphate medium with 10 mM in KH_2PO_4 was inoculated with a colony of the cells to be induced and grown at 30°C overnight on a shaker. Cells were collected by centrifugation and washed 2X in equal volumes of Lo P medium [Low Phosphate Medium (Lo P): 1X MOPS, 0.4% glucose, 0.15% vitamin free casamino acids (L59), 2 $\mu\text{g/ml}$ B1 (thiamine), 0.1 mM KH_2PO_4 , antibiotic
25 (ampicillin, 50-100 $\mu\text{g/ml}$) and resuspended in low phosphate medium (10 mls/tube). The cells were resuspended in the original volume in Lo P medium and then diluted 1:50 into fresh Lo P. The cells were incubated at 30°C for > 6 hrs to overnight on a shaker. The cells should achieve a final A_{600} of approximately 1.

Cells were removed by centrifugation at 10,000 rpm for 30 min. and washed once in 50 ml of PBS/NaN₃. They were stored at -2°C.

The pellets were resuspended into PBS/NaN₃, one pellet into 25 ml and the second pellet 40 ml. The first was refrozen at -20°C and thawed at 37°C a total of four times including the initial thaw. The second pellet, was sonicated four times of two minute duration. Both the freeze/thaw and sonication mixtures were centrifuged at 10,000 rpm for 30 minutes.

The following samples were saved for analysis by ELISA: A-cells in growth media, B-growth media from the first centrifugation, C-first centrifugation pellet PBS/NaN₃ wash, D-freeze/thaw pellet, E-freeze/thaw supernatant (25 ml), F-sonication pellet, and G-sonication supernatant (40 ml).

An SDS-PAGE nonreducing gel analysis was performed on samples A through G. Coomassie Blue staining indicated some Fab in samples A, D, E, F, and G. A c-erbB-2 ELISA of fractions A through G indicate activity in A, D, E, F, and G. However, the activity in fraction E was found to be higher than G. Similar purification procedures performed on 520C9 (non-humanized) expressed in *E. coli* also found the highest level of anti c-erbB-2 activity in fraction E.

To isolate Fab (containing the KT3 tagged H chain), a 2ml affinity column of Protein G Sepharose KT3 was washed with 3 column volumes of 0.1 M Na₂CO₃, pH 10.5, and then a large volume of PBS/NaN₃. A sample of each supernatant (20 ml of E and 35 ml of G) were separately passed over the same column. After the sample loading was complete the column was washed with 10 ml of PBS/NaN₃ which was added to the flow-through volume. The material bound to the column was eluted using 10 ml of 0.1 M Na₂CO₃, pH 10.5. The eluate was immediately neutralized and the protein concentration determined using Pierce Coomassie reagent: the sonicated eluted material from sample gave a concentration of 28 µg/ml and the freeze/thaw sample gave 7 µg/ml. SDS-PAGE analysis of the starting material, the flow through, and the eluate, shows there is still a large amount of the Fab in the flow through of both the freeze/thaw and the sonicated procedures.

Example 8

Renaturation Studies of Fab Produced in E. Coli

Renaturation of Fab produced in E. coli was successfully performed using procedures described in Buchner and Rudolph, Bio/Technology 9:157-162 (1991).

5 Using conditions described as optimal by Buchner and Rudolph humanized 520C9 Fab (TLW170-1) with binding activity to c-erbB-2 has been produced.

Furthermore, activity in the crude extract was obtained in the absence of denaturation/renaturation for two versions of the humanized 520C9 Fab (from clones 170-1, 169-1).

10

Methods

E. coli were grown overnight in 25 mls of HiP medium. The cells were harvested by centrifugation (10 minutes, 6,000 rpm in two 15 ml tubes), washed once in low phosphate medium. The cells contained in 1 tube (equivalent to 12.5 mls of the HiP medium) were used to inoculate the low phosphate culture, which
15 was grown at 30°C for 7 hours. The cells were harvested by centrifugation and combined into one 50 ml centrifuge tube. The cells were either frozen at -20°C overnight or treated with lysozyme.

The freshly collected or thawed cells were resuspended in 50 mls of 0.1 M Tris Cl, pH 7.8 containing 20 mM EDTA and 0.5 mg lysozyme/ml at room
20 temperature for 1 hour. The lysed cells were centrifuged at 20,000 rpm for 25 minutes and the supernatant stored frozen until they were assayed. Breakage was evident by the gel-like nature of the suspension. The pellets were washed with 50 mM Tris Cl, pH8, containing 20 mM EDTA. Sonication of one batch was used at this stage to break up the pellet and provide better extraction.

25 The pellets were resuspended in 50 mM Tris Cl, pH 7.8, containing 1 % Triton X-100, 0.5 M NaCl, and 20 mM EDTA. The pellets were collected by centrifugation and washed twice with 50 mM Tris Cl, pH 7.8, containing 20 mM EDTA. To the drained pellets were added 0.1 M Tris Cl, pH8, containing 6 M guanidine HCl, 0.3 M dithiothreitol, and 2 mM EDTA. The suspension was
30 vortexed occasionally at room temperature for 1 hour and the pellet collected by centrifugation at 40,000 rpm for 15-20 minutes. The pellet was reextracted with a

smaller volume of denaturant in one experiment.

The protein concentration was determined by Bradford analysis. SDS-PAGE was run on the samples with and without reduction to determine the purity and level of expression of Fab.

Renaturation was achieved by diluting the guanidine/DTE solution of Fab into 0.1 M Tris Cl, pH 8.2, containing 2 mM EDTA, 0.2 M L-arginine, and 2 mM oxidized glutathione. The final dilution of the Fab was 1:100, which produces a final concentration of 3 mM DTE in the redox system. The renaturation buffer was brought to 11°C before dilution was made.

Results and Discussion

Time Dependence of Renaturation of Pellet F:

A pellet (F) (see section VI for details), produced from 170-1 humanized 520C9 Fab in *E. coli* was extracted with guanidine and DTE and renatured as described above. The total protein concentration in the renaturation buffer was 35 and 18 µg/ml. Because the pellet during washing looked as if two layers were being separated during centrifugation, an attempt was made to separate them, resulting in two fractions. SDS-PAGE analysis did not indicate any differences between the two fractions.

To enable all of the assays to be done at once, dilutions from the guanidine/DTE solution of Fab (store at room temperature) were made at various times and renaturation started at 11°C (10°C was intended). Dilutions were performed by adding 100 µl of Fab/guanidine/DTE solution to 10 mls of renaturation buffer (which had been stored in the refrigerator). After 86 hours of renaturation, the samples were all assayed at the same time. The staggered times of renaturation were 86, 74.5, 38.5, 16.5 and 0 hours of renaturation. The buffer control produced no signal. Activity was detected in all samples. The best recovery of activity was seen with the longest time of renaturation. Because no positive control was possible, the extent of the recovery of activity could not be estimated.

These samples were assayed again about 3 days later. The activity was

again observed, but there was less difference between the shortest and longest times of renaturation. This was expected as all of the molecules reached the "plateau" region described by Buchner and Rudolph.

These experiments demonstrate that renaturation of Fab produced in E. coli was possible and that the humanized version of 520C9 refolded and was active.

Concentration of "Time Dependence" Samples:

About 6 days after the start of the renaturation studies, all the samples were pooled and concentrated on a stirred ultrafiltration device. The concentrate was stored at 4°C, and became cloudy.

Renaturation of Pellet F:

Most of the guanidine/DTE solution of pellet F was left after the time dependence studies described above. Therefore, the remainder was diluted 1:100 into renaturation buffer and incubated at 11°C. ELISA assays revealed activity.

Renaturation of TLW170-1:

The cells from 500 mls of medium induced for 7 hours at 30°C were treated with lysozyme and the supernatant collected by centrifugation. The pellet was extracted with 4 mls of guanidine/DTE. Because of the presence of DNA, the pellets resembled rubber before extraction. Bradford analysis showed that the amber colored 40,000 rpm supernatant contained 3.2 mg of protein/ml. The protein was diluted into 340 ml of renaturation buffer and incubated at 11°C. C-erbB-2 binding activity was observed by ELISA.

SDS-PAGE showed that little or no 50,000 MW Fab in the pellet was extracted by SDS sample buffer that did not contain reducing agent. However, a strong band having a molecular weight of about 25,000 was obtained when the pellet was extracted with SDS in reducing agent.

Comparison of Humanized and Murine Fabs:

169-1 and 170-1 are two different versions of humanized 520C9 Fab; 46-1

is the murine 520C9 Fab expressed in E. coli. Extraction of the cells obtained from 500 mls medium with 1 ml of guanidine/DTE gave total protein concentrations (measured by Bradford assay) of 2.78, 3.81, and 5.22 mg/ml, respectively. Extraction of the remaining pellet with an additional 0.75 ml of guanidine/DTE gave concentrations of 2.72, 1.92, and 3.32 mg/ml, respectively. The total samples (1.75 ml) were diluted separately into 175 mls of renaturation buffer and incubated at 11°C. Erb-binding activity was observed with all three samples.

Extraction of the pellet with SDS without reducing agent did not solubilize Fab produced by 169-1 and 170-1; however, the murine Fab, 46-1, was solubilized. All three Fabs were solubilized by SDS containing reducing agent.

Isolation of Fab:

The initially refolded Fab pellet, TLW 170-1 (which was concentrated to about 5 ml), developed some turbidity after concentration. A one ml sample was dialyzed against 20 mM Tris Cl, pH 8.3, overnight at 4°C. The sample was centrifuged at 100,000 x g for 30 minutes and the supernatant separated by chromatography using a Poros strong anion exchange column (0.5 ml fractions collected). When the sample was originally concentrated (before the turbidity developed), the total protein concentration was 240 µg/ml and when analyzed by nonreducing SDS-PAGE gel the Fab was just barely visible with Coomassie staining. By estimating the protein concentration based on the intensity of staining the yield of Fab was determined to be about 50 µg/ml. The entire sample was injected onto the anion exchange column and developed with a NaCl gradient in 20 mM Tris Cl buffer, pH 8.3. Peak tubes were analyzed by SDS-PAGE with silver stain. A murine Fab generated by papain digestion was used to identify the relative chromatographic elution time. However, gel analysis failed to show any FAB band in any of the peaks. An additional sample, however, showed good activity on the SKBr3 TNN plate coat assay.

Thus, both murine and humanized 520C9 Fabs are active and can be renatured from guanidine in a redox system.

Large samples of *E. coli* expressing murine 520C9 (46-1) have been grown at lower temperatures (23° and 27°C) to increase the amount of soluble Fab. A 10 liter sample was induced and grown at 27°C before the cells were collected. The periplasmic space was opened to release the soluble, secreted Fab localized to the periplasmic space. This material contained active, soluble Fab, as measured by SDS-PAGE and ELISA. The Fab obtained from the periplasmic space was concentrated and analyzed before it was passed over a column of immobilized c-erbB-2 extracellular domain. The column was washed and then eluted with a high concentration of LiCl (3.5M). From one-tenth of the "periplasmic Fab", about 400 µg of Fab was recovered. Fab that was not retained by the c-erbB-2 column was not active when assayed by a SDS-PAGE western blot.

Analysis of the murine 520C9 Fab (46-1) recovered from the affinity chromatography column showed essentially the identical results when compared to a proteolytically produced sample of 520C9 Fab in an ELISA assay. Therefore, it appears that the association constant of the recombinant Fab is the same, or nearly the same (within experimental error), as the Fab made from intact 520C9 antibody produced in ascites cells.

Example 9

Assays on Recombinant Humanized 520C9 Fab

The purpose of these experiments was to determine if humanized 520C9 Fab expressed in *E. coli* is capable of binding c-erbB-2.

Triplicate sets of uninduced and induced *E. coli* samples containing humanized 520C9 Fab (not refolded) were run on non-reducing SDS PAGE and western blotted onto a membrane. Two sets were probed with HRP-monoclonal anti-human kappa chain or with HRP-KT3, i.e., an antibody specific to the oligopeptide and TPPPEPET conjugated to horse radish peroxidase. A third set was probed with c-erbB-2 ECD-HRP. ECD-HRP is horse radish peroxidase conjugated to an antibody specific for c-erbB-2 (Nu2) ECD, an extracellular domain (ECD) of c-erbB-2 expressed in a secreted form from baculovirus infected SF9 insect cells (the Nu2 construct has the TPPPEPET polypeptide sequence

replacing the transmembrane domain of c-erbB-3. A number of nonspecific bands developed in both uninduced and induced lanes. One additional band with near the expected mobility for Fab may have developed only in the induced lanes, but was so close to another nonspecific band that it could be seen only as a widening of the lane.

Fab fragment generated from non-specific human IgG was bound directly to PVC microtiter wells and probed with Zymed™ goat anti-human antibody. Some signal was seen, but the signal was weak at the probe dilution used on the humanized Fab samples.

To avoid the uncertainties of using the goat anti-human probe, an ELISA was performed using wells coated with SK-Br-3 TNN cell extract, this extract is a source of cell expressed c-erbB-2 that does not bear the KT3 tag peptide. The presence of *E. coli* produced Fab bound to the cell extract was detected with KT3-HRP. A reassay of humanized Fab samples with this protocol showed activity in various fractions, with the highest activity in the freeze/thaw supernatant. Active Fab could not be quantitated in absolute terms for lack of a purified standard.

Refolded samples containing humanized 520C9 Fab were assayed with the SK-Br-3 TNN/KT3-HRP ELISA. Samples showed over a ten-fold increase in activity that occurred after 86 hours of refolding.

The refolded samples were reassayed with the same ELISA after an additional three days refolding time. Some further increase in activity was seen, but the ELISA curves flattened. Some samples refolded for longer times showed less activity; this could have been caused by proteolytic degradation among other causes. Samples from humanized clones 169-1 and 170-1 were active.

Humanized 520C9 Fab from both clones (169-1 and 170-1) is active. Refolding can increase activity more than 10- fold.

Biological Deposits

On March 24, 1992, Applicants have deposited with the American Type Culture Collection, Rockville, Md., USA (ATCC) the plasmid pLW187, described herein ATCC accession no. 68942. This deposit was made under the provisions

of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of patent procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

10 Equivalents

All publications and patents mentioned in the above specification are herein incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

WE CLAIM:

1. An immunoglobulin comprising, a humanized variable region, wherein said variable region is a member of a functional pair of variable regions specific for binding to c-erbB-2.
2. An immunoglobulin according to claim 1, said immunoglobulin further comprising, at least a portion of a human immunoglobulin constant region.
3. An immunoglobulin according to claim 2, wherein said constant region is a complete constant region.
4. An immunoglobulin according to claim 2, wherein said humanized variable region and said constant region are of the same chain type.
5. An immunoglobulin according to claim 4, wherein said chain type is heavy chain.
6. An immunoglobulin according to claim 4, wherein said chain type is light chain.
7. An immunoglobulin molecule according to claim 1, wherein said immunoglobulin further comprises in operable combination, a leader sequence.
8. An immunoglobulin according to claim 2, said immunoglobulin further comprising in operable combination, an oligopeptide tag, wherein said tag is joined to said constant region.
9. A multi-polypeptide immunoglobulin, comprising an immunoglobulin according to claim 1.

10. A multi-polypeptide immunoglobulin according to claim 9, comprising in operable combination,
 - a) a first immunoglobulin molecule comprising, a first humanized variable region, wherein said variable region is a member of a functional pair specific for c-erbB-2, and
 - b) a second immunoglobulin molecule comprising, a second humanized variable region, wherein said second variable region is a member of the same functional pair as said first immunoglobulin variable region.
11. A multi-polypeptide immunoglobulin according to claim 10, wherein said first immunoglobulin molecule further comprises a first constant region, and said second immunoglobulin molecule further comprises a second constant region.
12. A multi-polypeptide immunoglobulin according to claim 11, wherein said first constant region of said is a heavy chain constant region, and said second constant region is a light chain constant region.
13. An immunoglobulin according to claim 12, wherein said first variable region and said first constant region belong to the same chain class, wherein said second variable region and said second constant region belong to the same chain class.
14. A multi-polypeptide immunoglobulin according to claim 12, said aggregate further comprising a therapeutic moiety.
15. A multi-polypeptide immunoglobulin according to claim 14, wherein said therapeutic moiety is selected from the group consisting of, toxins, radionuclides, radio-opaque imaging compounds, enzymes, drugs, and pro-drugs.

16. A nucleotide sequence, said nucleotide sequence encoding a first humanized variable region sequence, wherein said variable region is a member of a functional pair specific for c-erbB-2.

17. A nucleotide sequence according to claim 16, said sequence further comprising at least a portion of a human constant region.

18. An expression vector, said vector comprising in operable combination, a promoter sequence, operably joined to a nucleotide sequence according to claim 16.

19. A nucleotide sequence comprising a first nucleotide sequence according to claim 16, a second nucleotide sequence according to claim 16, wherein when the variable regions encoded by said nucleotide sequences of form a functional pair specific for c-erbB-2.

20. A therapeutic composition comprising, an effective amount of a multi-polypeptide immunoglobulin according to claim 9.

21. A cell transformed with a vector according to claim 18.

22. A method of detecting carcinoma cells, said method comprising the step of, administering a compound according to claim 1 to a patient.

23. A method of treating a cancer patient, said method comprising the step of administering a compound according to claim 1 to said patient.

FIGURE 1

COMPLETE NUCLEOTIDE SEQUENCE OF
HUMANIZED ANTI-erbB2 520C9 Fab LW218 [H-28;L-38]

```
1 CTCTCATAAA GTTGTACCGG GCGAGAGTTA TAATCGCTTT GTTTTATTT
81 TTTAATGTAT TTGTACATGG AGAAAATAAA GTGAAACAAA GCACTATTGC
101 ACTGACACTC TTACCGTTAC TGTTTACCCC TGTGACAAA GCGGAGATGC
131 AACTGATGGA GTCTCGCCCT CAGCTCAACA AAGCTCGGGG GTCAATGAG
201 GTCTCGTCCA AGCTTCTCTG TTACACCTTT ACCAAGTATG GAATGAACTG
231 GTTCCGACAG GCGGCTGAGC AAGCGCTTCA CTGATGCGA TCCATAAACA
301 COTACACTGG ACACTCAACA TATGCTGATG ACTTCAAGGA AAGAGTCACC
381 ATGAGCAGAG ACACATGAGG GAGGACAGCG TACATGAGCG TGAAGAGCGT
401 GAGATCTGAC GACAGCGGCG TGTATTACTG TCCGACACCA TTGCGCTTG
481 GTTACTGAGG CCAAGGAGCC CTGGTCACCG TGTGCTCAGC GTCCACCAAG
501 GCGCATGAGG TGTTCGCGCT GCGACGCTCC TCGAAGAGCA CCTCTCGCGG
531 GACAGCGGCG CTGAGCTGCG TGTCAAGCA GTACTTCGCG GAGCGGCTGA
601 CGCTGCTGCG GAACTCAGCG GCGCTCAGCA GCGCGCTGCA GAGCTTCGCG
681 GCTGCTGAGG AGTCTCAGG AGTGTACTCG GTGAGCAGCG TGTGACCGT
701 GCGCTGAGG AGCTTGGCGA GCGAGAGCTA CATCTGCAAG GTGAAATGCA
781 AGCGCAGCAA CAGCAAAGTG GAGAAAGAAA GTAGTAGCGG GCGCGCGGAA
801 GCGGAAAGCT AATAGATGCA TCGAGAAAT AAAATGAAAC AAGCACTAT
831 TCGAGTGGCA CTCTTAAGCT TACTGTTTAC GCGTGTGACA AAGTCCACA
901 TCGAGATGAC CCAATCTCCA TCCTCCCTGT CTGATCTGT AAGACACAGA
931 GTCAAGATCA GTTGGCGGCG AAGTCAAGAG ATTGGTAATA GCTTAAGCTG
1001 GTATGAGCAG AAAAGAGGGA AAGCGGCTAA GGTGCTGATC TACGCGCAGT
1031 GCAATTTAGA TTCTGGGCTG GATCAAGGT TCAATGCAAG TCGATCTGGG
1101 AGAGATTTTA GTTTCACCAT CAGGAGTCTG CAGCGTGAAG ATATTGCAAC
1181 ATATTACTGT CTACAATATG GTATTTTTCG GTAGAGCTTC GCGCAAGCGA
1201 CAGGAGTGA GATTAAACGA ACTGTGCGTG CAGCATGTGT GTTCACTTG
1281 GCGGATCTG ATGAGCAATG GAAATCTGGA AGTGGCTCTG TTGTGTGCTT
1301 GGTCAATTAAC TTCTACCCCA CAGAGGCCAA AGTACAGTGG AAGGTGAGTA
1331 AGCGGCTCCA ATCGGATTAAC TCGCAAGAGA GTGTCAAGGA GCAAGACAGC
1401 AAGGACAGCA CCTACAGCGT CAGCAGCAGC GTGAGGCTGA GCAAGCAGCA
1481 GTACGAGAAA CACAAAGTCT AGCGCTCCCA AGTCACCCAT CAGCGGCTCA
1501 GTTGGCGGCT CACAAAGAGC TTCAACAGCG GAGAGTGTTA ATCTCCACCG
1551 ATCG
```

FIGURE 2

PCR STRATEGY FOR FUSING MOUSE 520C9 CDRs TO HUMAN FRs

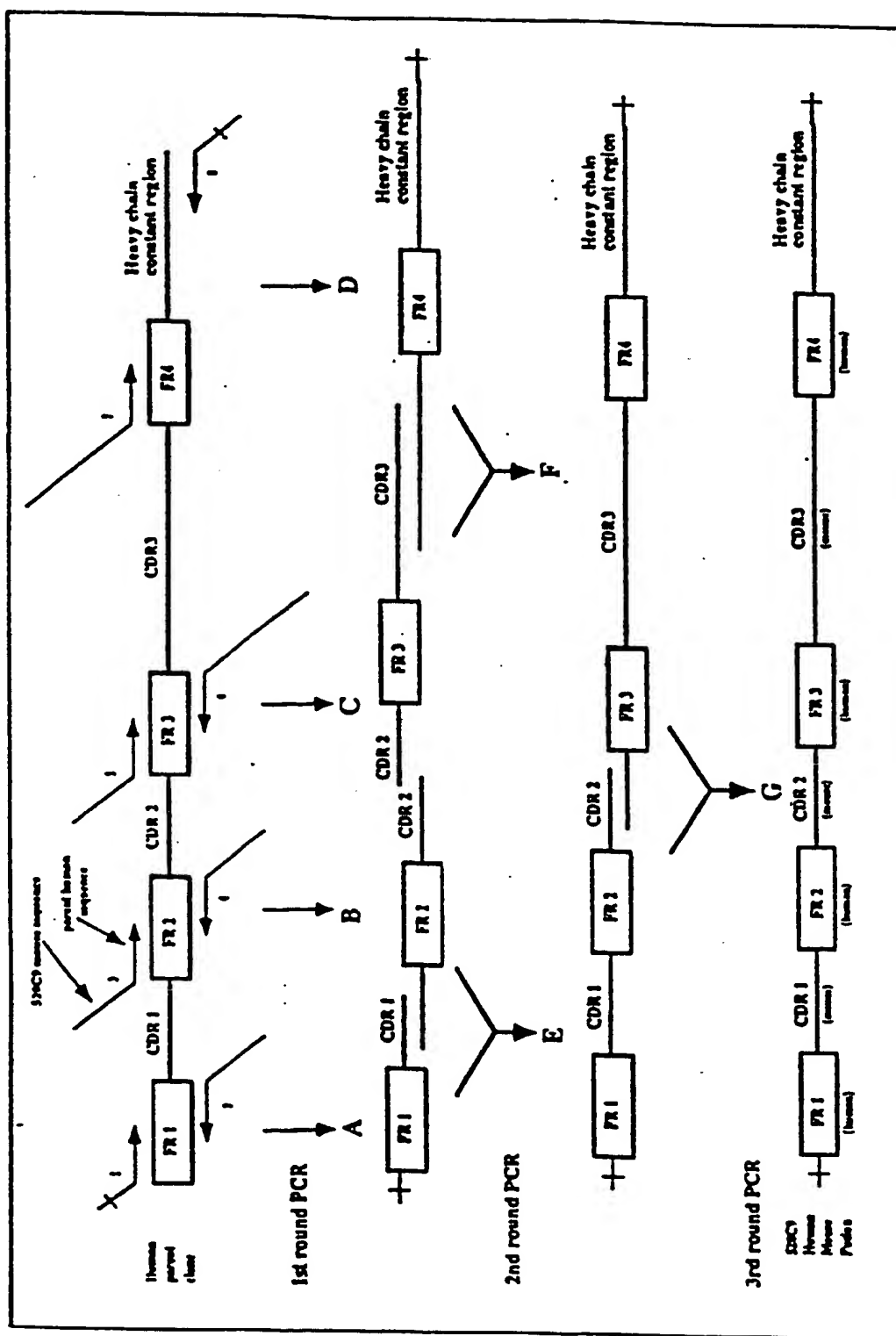
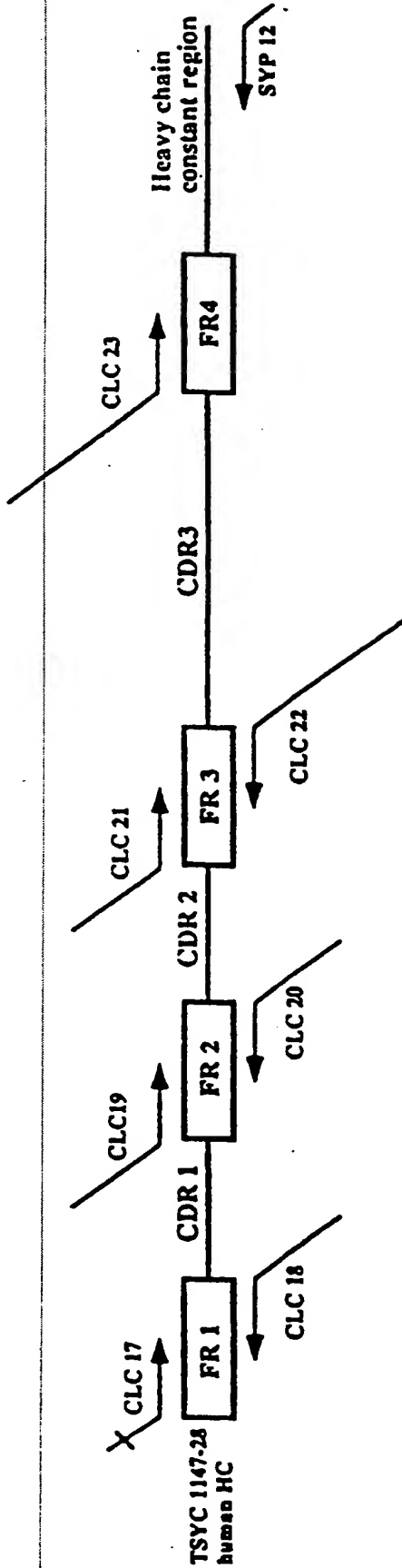


FIGURE 3
DESIGN AND LOCATION OF PRIMERS FOR 520C9 HEAVY CHAIN PCR FUSION



	mouse CDR	human FR
CLC 17	GGCTGCTCGAG	TCTGGGCCCTGAGGT
CLC 18	GTTTCATTCCATAGTT	GGTAAAGGTGTAACCAGAAG
CLC 19	AACTATGGAATGAAC	TGGGTGCGACAGGCCCCCTGG
CLC 20	CATCAGCATATGTTGACTGTCCAGTGTAGGTGTTTATCCA	TCCCATCCACTCAAGCCCTT
CLC 21	CACCTACACTGGACAGTCAACATATGCTGATGACTTCAAGGAA	AGAGTCACCATGACCACAGA
CLC 22	GTAAGCAAAACCCAAATCG	TCTCGCACAGTAATACACGG
CLC 23	CGATTGGGTTTGCTTAC	TGGGGCCAGGGAACCCTGGT
SYP 12	CTGGCACTAGT	AACTTTCTTGTCCACCTTGGTG
	Xho I	Spe I

FIGURE 4
DESIGN AND LOCATION OF PRIMERS FOR 520C9 LIGHT CHAIN PCR FUSION

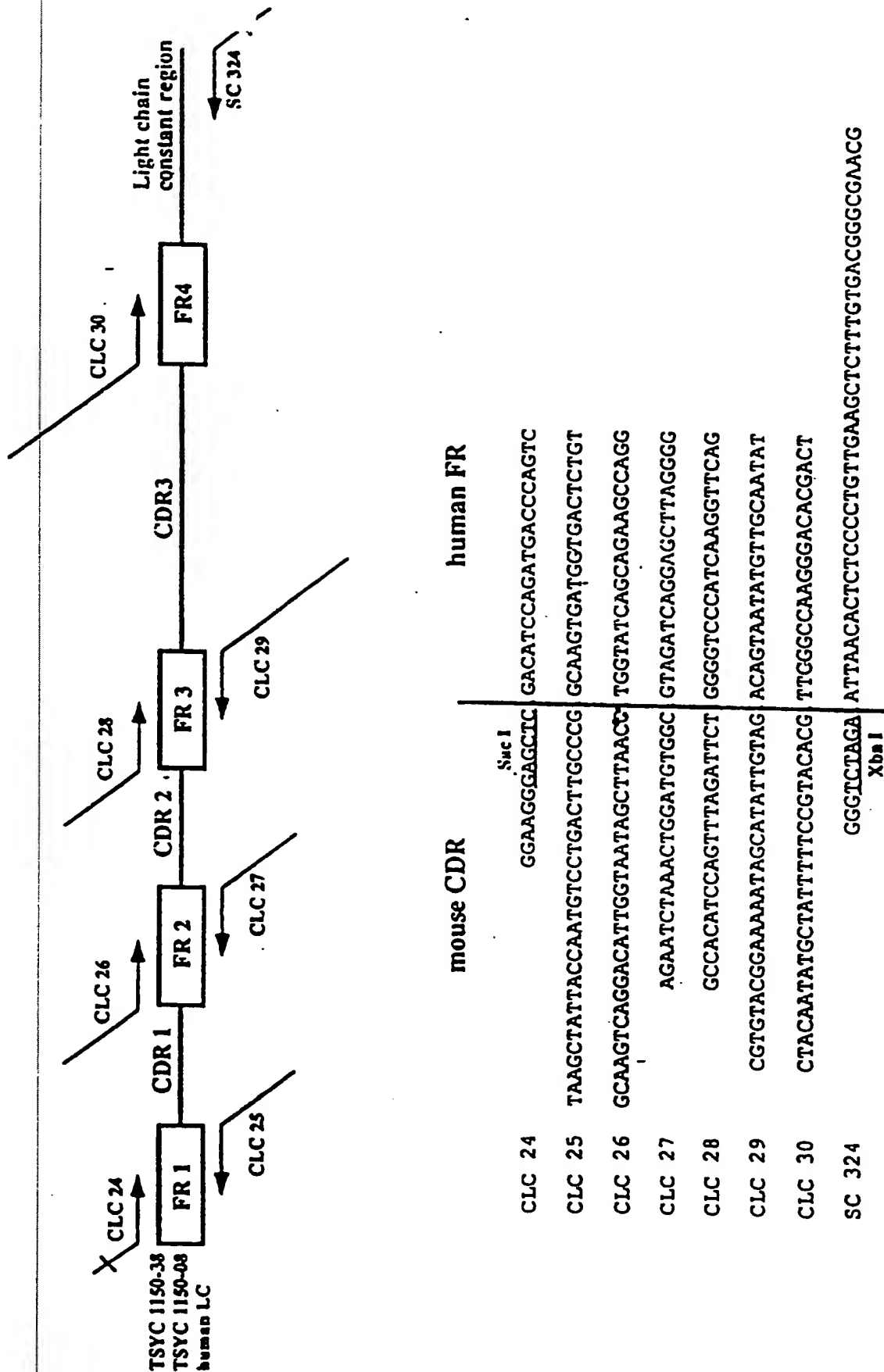


FIGURE 5

PRIMERS FOR INTRODUCING ANTIBODY SEQUENCES INTO A pBR BASED EXPRESSION VECTOR

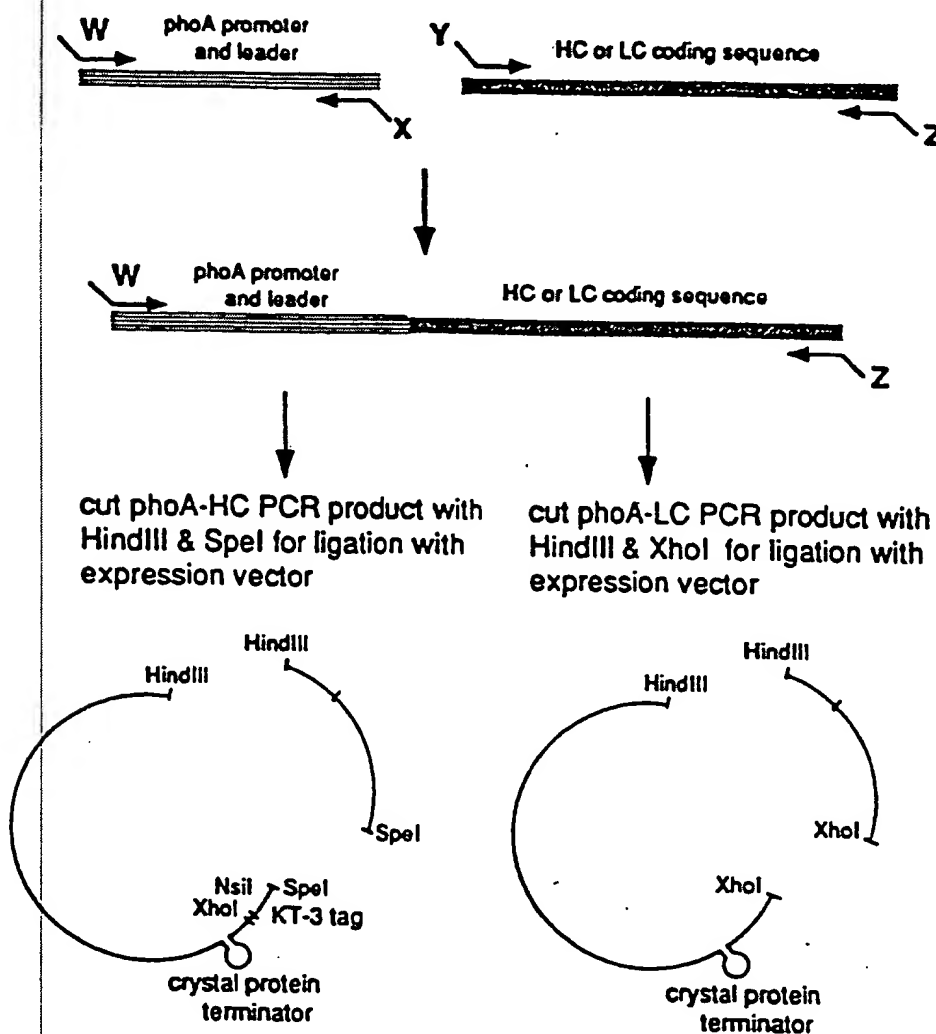
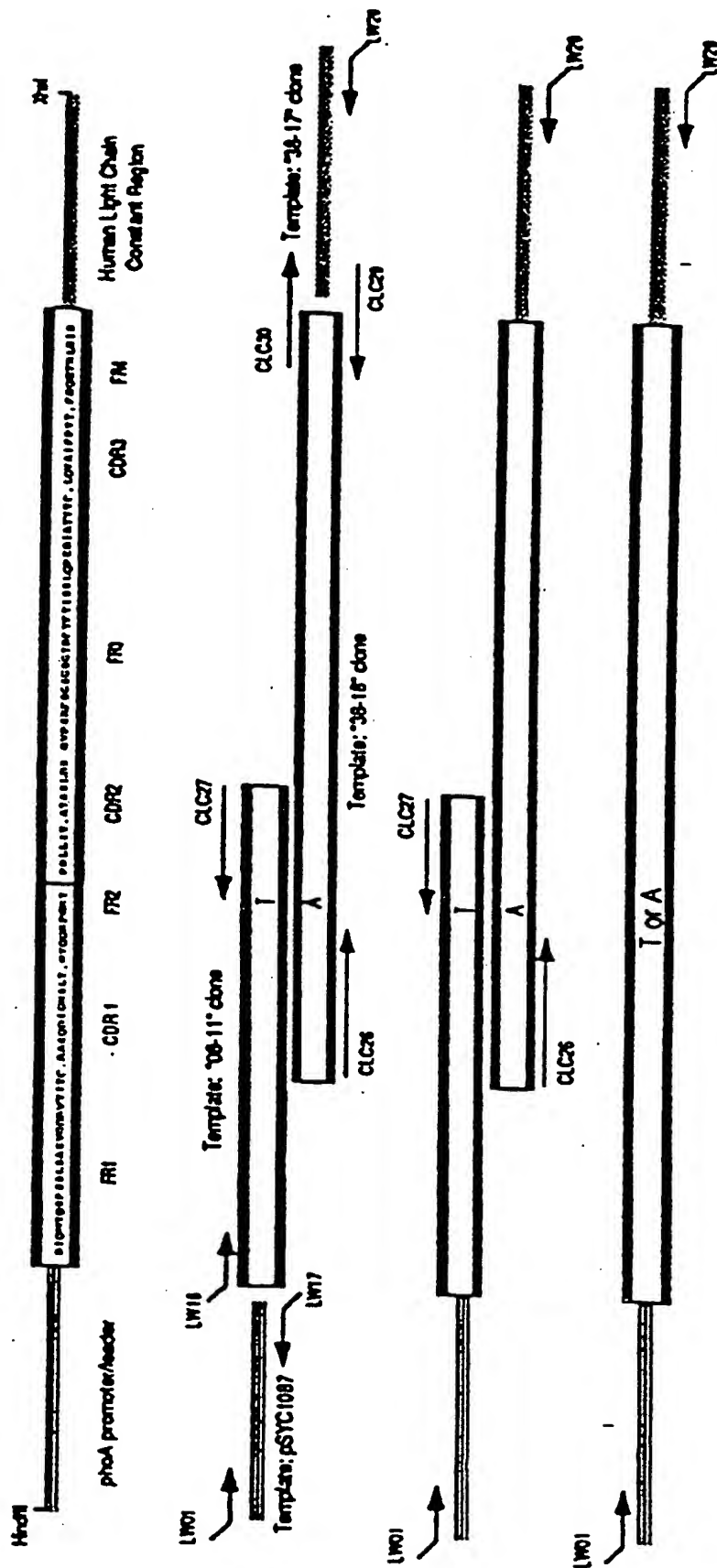


FIGURE 6

ALTERED STRATEGY FOR OVERLAP PCR OF HUMANIZED LIGHT CHAIN

Desired PCR product



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/03080

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12N15/13; C12P21/08; C12N15/63; A61K39/395 A61K49/00						
II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px; margin: 5px 0;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; padding: 2px;">Classification System</th> <th style="width: 75%; border: 1px solid black; padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">Int.Cl. 5</td> <td style="border: 1px solid black; padding: 5px;">C12N ; C12P ; A61K</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched⁸</div>			Classification System	Classification Symbols	Int.Cl. 5	C12N ; C12P ; A61K
Classification System	Classification Symbols					
Int.Cl. 5	C12N ; C12P ; A61K					
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹						
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claims No. ¹³				
X	BIO/TECHNOLOGY vol. 9, no. 12, December 1991, NEW YORK, US pages 1373 - 1377 L. GARRARD ET AL. 'Fab assembly and enrichment in a monovalent phage display system.' see abstract <div style="text-align: center;">---</div>	1,2,4,6, 7,9, 16-18				
Y	NATURE vol. 332, 24 March 1988, LONDON, GB pages 323 - 327 L. RIECHMANN ET AL. 'Reshaping human antibodies for therapy.' see the whole document <div style="text-align: center;">---</div> <div style="text-align: right;">-/--</div>	1-15,20, 22,23				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document not published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">29 JUNE 1993</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">13. 07. 93</div>					
International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">NOOIJ F.J.M.</div>					

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claims No.
Y	WO,A,8 910 412 (APPLIED BIOTECHNOLOGY, INC. & WHITEHEAD INSTITUTE FOR BIOMED. RESEARCH) 2 November 1989 see page 22, line 22 - line 31; claims ---	1-15, 20, 22, 23
A	WO,A,8 503 523 (CETUS CORPORATION) 15 August 1985 see claims ---	1-23
P, X	BIOCHEMISTRY vol. 31, no. 24, 23 June 1992, WASHINGTON DC, US pages 5434 - 5441 R. KELLEY ET AL. 'Antigen binding thermodynamics and antiproliferative effects of chimeric and humanized anti-p185HER2 antibody Fab fragments.' see abstract -----	1-13, 16-19, 21

INTERNATIONAL SEARCH REPORT

national application No.

PCT/US 93/03080

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 22 and 23 are directed to a diagnostic method and to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9303080
SA 73263

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

29/06/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8910412	02-11-89	AU-A- 3568289	24-11-89
		EP-A- 0412116	13-02-91
		JP-T- 3505964	26-12-91
WO-A-8503523	15-08-85	US-A- 4753894	28-06-88
		CA-A- 1253090	25-04-89
		EP-A,B 0153114	28-08-85
		JP-T- 61500789	24-04-86
		US-A- 5169774	08-12-92